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Award Number: DAMD17-98-1-8026

TITLE: The Role of Steroid Receptor Coactivator-1 in Breast Cancer

PRINCIPAL INVESTIGATOR: Neil McKenna, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: June 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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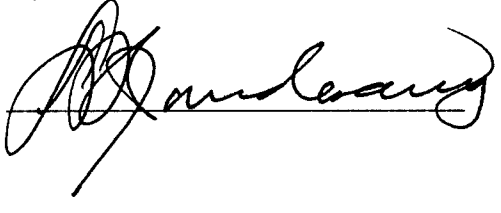

04/20/01

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2000	Annual Summary (18 May 99 - 17 May 00)

4. TITLE AND SUBTITLE
The Role of Steroid Receptor Coactivator-1 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8026

6. AUTHOR(S)
Neil McKenna, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-MAIL:
nmckenna@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jun 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The steroid hormones estrogen and progesterone and their intracellular receptors, ER and PR, have been implicated in the development and progression of primary epithelial breast cancer. Regulation of gene expression by liganded steroid receptors is mediated by members of the steroid receptor coactivator (SRC-1) family, which are required by steroid receptors for efficient transcriptional regulation. Targeted deletion of SRC-1 in our laboratory suggests that it is required for hormone-stimulated proliferation of the mammary gland, implicating SRC-1 as a potential therapeutic target in breast cancer. For this reason we set out to examine the molecular role of SRC-1 in breast cancer. Using the liganded PR as a bait in a biochemical interaction assay, we identified an SRC-1 containing complex from cancer cell nuclear extracts. This complex was recruited by human PR in a ligand-dependent manner to acetylate core histones, a step thought to be a key event in the activation of steroid-regulated genes. We are currently identifying other members of this active SRC-1 complex. We suggest that SRC-1 and SRA function in breast cancer as a member of a large multiprotein complex which is recruited stably by liganded steroid receptors for efficient transcriptional regulation, and that members of the active SRC-1 complex are potential therapeutic targets in breast cancer.

14. SUBJECT TERMS
Breast Cancer, Steroid Receptor coactivators, steroid hormone receptors, steroid hormones, gene expression

15. NUMBER OF PAGES
36

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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The Role of Steroid Receptor Coactivator-1 in Breast Cancer

Annual Summary Report, 1999-2000

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Introduction

Steroid hormone action is mediated by members of the nuclear receptor superfamily, the largest group of metazoan transcription factors, regulate their cognate genes in a ligand-inducible manner (Tsai and O'Malley, 1994). Nuclear receptor coactivators are molecules that interact with nuclear receptors and enhance their transactivation (McKenna *et al.*, 1999). Most coactivators are, by definition, rate-limiting for nuclear receptor activation and have multiple functions, including direct interactions with basal transcription factors and covalent modification of histones and other proteins. Key questions in the study of breast cancer focus upon (i) the exact mode of action of nuclear receptor coactivators and (ii) their role at the molecular level in the genesis and progression of breast tumors.

Perhaps the best characterized family of nuclear receptor coactivators is the SRC family, named after the initial cloning in our laboratory of steroid receptor coactivator-1 (SRC-1), and currently known to include two other members, TIF2/SRC-2 and RAC3/SRC-3. Targeted deletion of SRC-1 in our laboratory suggested that it is required for hormone-stimulated proliferation of the mammary gland, implicating SRC-1 as a potential therapeutic target in breast cancer (Xu *et al.*, 1998). The remarkable growth rate of studies on the SRC family has emphasized the complexity of the relationships between coactivators and their cognate receptors.

As the project has progressed we have become convinced any consideration of the role of SRC-1 in a disease as complex as breast cancer must account for the fact, as I have shown, that SRC-1 exists in a multiprotein complex. For example, targeted deletion of the SRC-1 gene in mice results in a compensatory overexpression of another family member of TIF2/SRC-2, a probable contributing factor in the unanticipated stable phenotype of the SRC-1 null mutant mouse (Xu *et al.*, 1998). TIF2 was shown in my previous work to exist as a member of the active SRC-1 complex in breast cancer cells, a fact which gives some insight into its overexpression in SRC-1 null mutant mice.

For these reasons, the natural compass of the project extended to the role in breast cancer etiology not only of SRC-1, but also that of factors characterized as members of the SRC-1 complex. In particular, we

focussed upon the mechanism whereby SRC-1 exerts its effects on steroid receptor regulation of gene expression in breast cancer cells. Fundamental questions posed at the outset were (i) what are the other members of the active SRC-1 complex in cancer cells, (ii) what is the functional significance of this complex and (iii) how might these factors contribute to the development of breast cancer? By addressing these issues, we sought to sketch a broad picture of the potential role of SRC-1 and its associated factors in the development of breast cancer.

Body

The SRC-1 complex: a target of liganded PR in breast cancer cells

A crucial part of the study was to show that the active SRC-1 complex was recruited by an activated steroid receptor. Our initial experiments set out to establish that SRC-1 was recruited by functionally active steroid receptors in cancer cells. Nuclear extracts were prepared from cultured cancer cells and incubated in the presence and absence of progesterone with a recombinant GST fusion of the progesterone receptor A-form purified from baculovirus-infected Sf9 cell pellets. PR-interacting molecules were identified by probing western blots of PR-recruited complexes using antibodies specific for selected coactivators (Appendix 1A). SRC-1 was recruited by GST-PR-A in a ligand-dependent manner from nuclear extracts, as was the SRC member RAC3/SRC-3. Surprisingly, we failed to observe significant recruitment by PR in the presence of ligand of the nuclear receptor "cointegrator" CBP, previously identified in our laboratory as a factor required by PR and ER for efficient transcriptional activation (Smith *et al.*, 1996). Furthermore, GST-PR-A failed to recruit SRC-1 in the presence of the antihormone RU486 (Appendix 1B). RU486 has been used with some success in the treatment of advanced breast cancer (Mahajan and London, 1997). While the clinical significance of this result remains to be determined, it is tempting to speculate that its clinical role may be in uncoupling the interaction of PR with the active SRC-1 complex.

Progesterone receptor recruits SRC-1 acetyltransferase activity in a ligand-dependent manner

An indispensable step in steroid hormone regulation of target genes is thought to be the disruption of chromatin structure at the promoters

of these genes by targeted modification of nucleosomal histones, including acetylation. Several studies have implicated the state of acetylation of nucleosomal histones as a potential etiological factor in breast cancer (Pasqualini *et al.*, 1989; Siddique *et al.*, 1998). Recent data have characterized intrinsic acetylation functions in several nuclear receptor coactivators, implicating them as potential catalytic intermediaries in this process (Spencer *et al.*, 1997). For these reasons we decided to determine whether PR might recruit acetylase activity from cancer cell nuclear extracts. We performed a protein acetylation assay, using core histones as substrates in reactions catalyzed by PR-recruited factors in cancer cell nuclear extracts. Acetylase activity targeting core histones associated with PR in a ligand-dependent manner (Appendix 2A). Significantly, this acetylase activity did not require recruitment of CBP. These experiments identified SRC family members as primary functional targets of liganded PR in cancer cells. With this result in mind, the clinical significance of the uncoupling of the PR-SCR-1 interaction by RU486 (Appendix 1B) may lie in its ability to prevent PR from properly configuring the nucleosomal structure at its cognate promoters.

Characterization of the SRC-1 complex in cancer cells

Having observed that the affinity bait GST-PR-A purified SRC-1 in a ligand-dependent manner, we decided to attempt to scale this procedure up towards a level of purification and yield that would permit sequencing of individual proteins. By doing this, we hope to establish the identity of the molecular partners of SRC-1 *in vivo*, which might lead to new therapeutic targets in breast cancer. This would be of particular importance since we had previously shown that RU486, a drug used in breast cancer treatment, prevents the interaction of the SRC-1 complex with PR.

The silver stain gel (Appendix 2B) shows the highest level of purity which we have been able to achieve using our affinity approach. For receptor pull downs (PDs), GST-PR-A was bound to glutathione-coupled sepharose beads for one hour at prior to extensive washing with 0.4M KCl buffer D and a final rinse with buffer D. Nuclear extracts were adjusted to 0.18M KCl and incubated with sepharose-bound GST-PR-A for 2-3h at 4°C prior to washing with 0.18M KCl buffer D and elution by boiling in SDS-sample buffer. Samples were electrophoresed on a 7.5%

for 2-3h at 4°C prior to washing with 0.18M KCl buffer D and elution by boiling in SDS-sample buffer. Samples were electrophoresed on a 7.5% SDS-PAGE gel prior to protein detection by silver staining. A series of polypeptides can be seen interacting with the PR in a ligand-dependent manner. We are currently working towards establishing the identity of the proteins, one of which we have established is SRC-1 (see Appendix 1).

Training as a scientific communicator

During the past year, I have sought to consolidate the experience which I gained as a scientific communicator in my first year as a US Army Postdoctoral Fellow. A review article was published in the *Journal of Steroid Biochemistry and Molecular Biology*, and a second review will appear in the book series *Molecular Regulation*. I saw the 2nd Era of Hope Meeting in Atlanta as an excellent opportunity to publicize the work of the Department of Defense Breast Cancer Research Program. For this reason I sought permission from the DOD to summarize selected proceedings of the meeting for inclusion in an commentary article to be submitted to a medical journal. Having such an article published would be of great benefit not only to me as a breast cancer researcher but, more importantly, would also bring the work of the DOD Breast Cancer Research Program to the attention of a wider audience. Several medical journals were contacted in connection with the article and we received a positive response from *Nature Medicine*. A manuscript has been submitted and their decision is awaited.

Difficulties encountered

A key problem has been two unique features of the SRC-1 molecule: the instability and toxicity in eukaryotic cells. SRC-1 rapidly degrades after cell lysis, to such an extent that the full length cannot be routinely produced in baculovirus cells without significant proteolysis. As a result we have been forced to re-evaluate our approach to characterizing expression patterns of SRC-1 in breast cancer. The toxicity of the protein in tissue culture cells in which we attempted to overexpress the full-length and dominant -negative forms of the protein meant that tissue culture cells overexpressing SRC-1 were subject to negative selection, which hampered our efforts to select appropriate clones. This is an ongoing problem.

Key accomplishments

- The SRC-1 complex is recruited by an active steroid receptor from breast cancer cell nuclear extracts (unpublished).
- Progesterone receptor (PR) recruits protein acetyltransferase activity from breast cancer cell lysates (unpublished).
- RU486, a drug used in the treatment of breast cancer, uncouples the interaction between PR and the SRC-1 complex
- A multiprotein SRC-1 complex was affinity-purified from cancer cell extracts using a GST-tagged recombinant PR (unpublished).
- Two reviews were submitted to and accepted by the *Journal of Steroid Biochemistry and Molecular Biology* and a book chapter was written for the series Molecular Regulation (Humana Press) (Appendix 3).
- Commissioned by *Nature Medicine* to author a commentary summarizing current developments in the role of nuclear receptors and breast cancer, as discussed at the recent Department of Defense 2nd Era of Hope Breast Cancer Research Program Meeting in Atlanta, GA (unpublished).

Reportable Outcomes

McKenna, N.J., Barron, N.W., Lanz, R.B. and O'Malley, B.W. (2000). The molecular role of the steroid receptor coactivator-1 complex in breast cancer. 2nd Era of Hope Meeting of the Department of Defense Breast Cancer Program. Atlanta, GA, USA.

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McKenna, N.J., Nawaz, Z., Tsai, S.Y. and Tsai, M.-J. (2000). Coactivators and corepressors. In: Conn, P.M. & Means, A.R. (eds) Molecular Regulation, Humana, New Jersey. pp. 385-394.

Conclusions

My tenure as a Department of Defense Postdoctoral Fellow over the past year has enabled my training as a breast cancer researcher to continue to progress in two main areas. Firstly, in our experimental work, we are continuing to steadily construct a coherent model of the role of SRC-1 in breast cancer. The most important accomplishment has been to show that the SRC-1 is an important functional target of progesterone receptor in cancer cell nuclear extracts. A source of difficulty has been the challenge of purifying this complex to a purity and yield sufficient to enable direct sequencing, and we hope to address this problem. In addition, our group is continuing to address the extent of overexpression of a member of the SRC-1 complex, SRA, in breast tumors.

In the second area of development, I am learning the importance of writing regular reviews and commentaries in the field of breast cancer as it relates to my work. I foresee this role assuming greater importance as my career progresses and I move into a more senior research position. The benefits of this are twofold: firstly, it requires one to keep up with the literature in the field, a task which would be otherwise difficult to achieve. Secondly, keeping our peers informed of our perspectives on the direction of the field ensures the dissemination and exchange of views which will drive innovative thinking and collaboration, as we strive towards an understanding of the molecular basis of breast cancer.

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Spencer, T.E., Jenster, G., Burcin, M.M., Allis, C.D., Zhou, J.X., Mizzen, C.A., McKenna, N.J., Onate, S.A., Tsai, S.Y., Tsai, M.-J. and O'Malley, B.W. (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature*, 389, 194–198.

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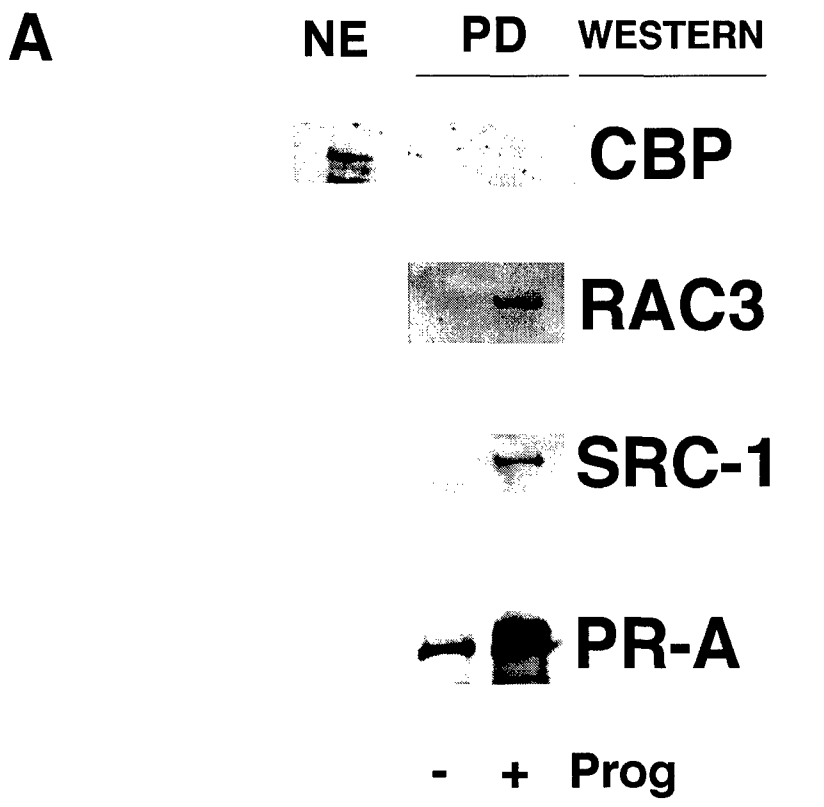
Appendices



APPENDIX 1

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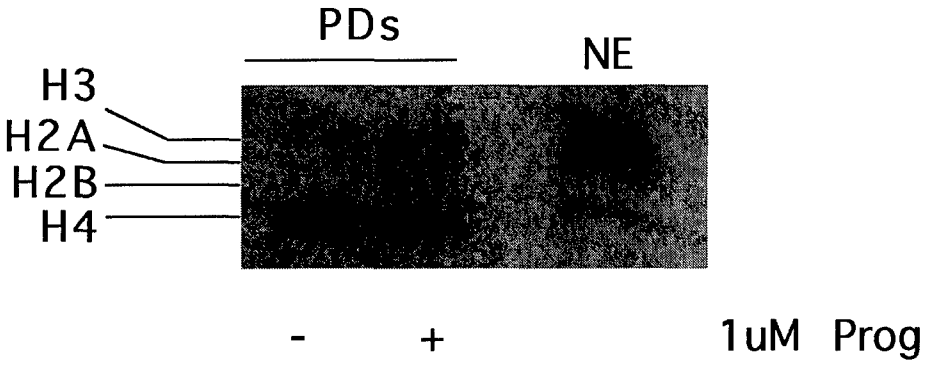

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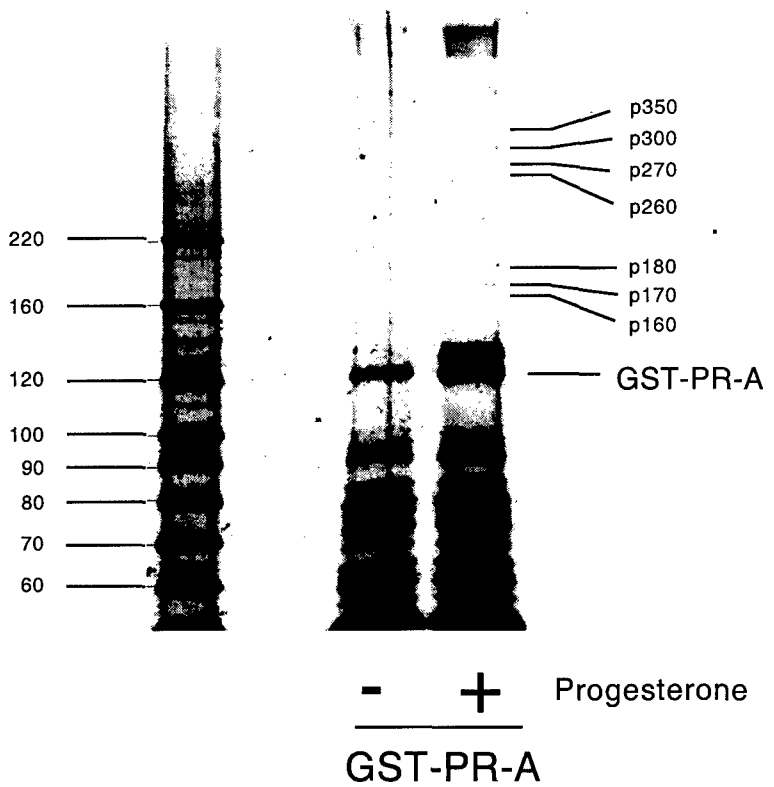

APPENDIX 2

PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 69 (1999) 3–12

The Journal of Steroid Biochemistry & Molecular Biology

Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions☆

Neil J. McKenna, Jianming Xu, Zafar Nawaz, Sophia Y. Tsai, Ming-Jer Tsai, Bert W. O'Malley *

Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, U.S.A.

Abstract

Nuclear receptors are ligand-inducible transcription factors which mediate the physiological effects of steroid, thyroid and retinoid hormones. By regulating the assembly of a transcriptional preinitiation complex at the promoter of target genes, they enhance the expression of these genes in response to hormone. Recent evidence suggests that nuclear receptors act in part by recruiting multiple coregulator proteins which may have specific functions during transcriptional initiation. Liganded receptors recruit members of the SRC family, a group of structurally and functionally related transcriptional coactivators. Receptors also interact with the transcriptional cointegrators p300 and CBP, which are proposed to integrate diverse afferent signals at hormone-regulated promoters. p300/CBP and members of the SRC coactivator family have intrinsic histone acetyltransferase activity which is believed to disrupt the nucleosomal structure at these promoters. Other nuclear receptor coactivators include a member of the SWI/SNF complex, BRG-1, which couples ATP hydrolysis to chromatin remodelling, and the E3 ubiquitin-protein ligases E6-AP and RPF-1. Finally, nuclear receptor coactivators appear to be organized into preformed subcomplexes, an arrangement that may facilitate their efficient assembly into diverse higher order configurations.

Keywords: Nuclear receptors; Coactivators; Enzymes; Proteins

1. Introduction

Steroid, thyroid and retinoid hormones control eukaryotic gene expression patterns by interaction with a group of intracellular ligand-inducible transcription factors which comprise the nuclear hormone receptor superfamily [1]. This superfamily is the single largest class of eukaryotic transcription factors and its members mediate signaling pathways in a wide variety of physiological systems. The superfamily is broadly divisible into three subclasses: the type I receptors for steroid hormones, including progestins (PR), estrogens (ER), androgens (AR), glucocorticoids (GR) and mineralocorticoids (MR); the type II receptors for thyroid hormone (TR), vitamin D (VDR), 9-*cis* (RXRs) and all-*trans* retinoic acid (RARs) and those for which cognate ligands have not yet been characterized, the orphan receptor subclass.

Recently, the cloning and functional characterization of coregulator proteins which interact with nuclear receptors to effect an efficient transcriptional response has gained increasing attention. Broadly speaking, these proteins fall into one of two classes: coactivators and their associated proteins, and corepressors and their associated proteins. This brief review, while summarizing some of the most significant advances in this area in recent years, will emphasize the role of coactivators in receptor action.

2. Overview of receptor action

Nuclear receptors undergo a series of functionally well defined steps which culminate in target gene activation (Fig. 1). Interaction between ligand and recep-

Proceedings of Xth International Congress on Hormonal Steroids, Quebec, Canada, 17–21 June 1998.

* Correspondence to B. W. O'Malley. Tel.: 713 798 6205; fax: 713 798 5599; e-mail: berto@bcm.tmc.edu

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PII: S0960-0760(98)00144-7

APPENDIX 3

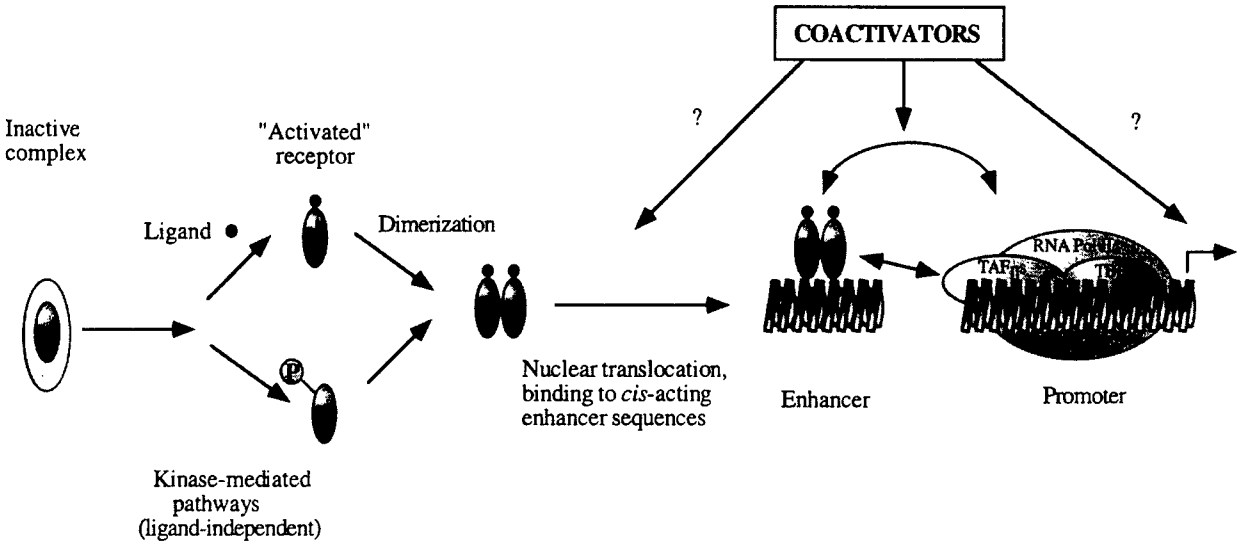

Fig. 1. Overview of events initiated by binding of ligand to nuclear receptor and culminating in transcriptional activation of a hormone-regulated gene. 'Activation' of receptor occurs either through hormone binding or by catalytic phosphorylation by kinases of specific residues on the receptor. Specific conformational alterations result in dimerization, nuclear translocation and apposition to a specific *cis*-acting hormone response element. The precise mechanism of assembly of a stable preinitiation complex composed of basal transcription factors and other promoter-specific factors is unknown. While receptor can directly contact general transcription factors, many coactivators are known to mediate these interactions. In addition, they are likely to play multiple roles in both processes which precede DNA binding and those which follow formation of the transcriptional preinitiation complex. Abbreviations: TBP, TATA box-binding protein; TAF_{II}s, TBP-associated factors; GTFs, general transcription factors; RNA pol II, RNA polymerase II.

tor occurs in two ways: (i) either in the cytoplasm or nucleus of target cells, inducing nuclear translocation of the ligand–receptor complex (for type I receptors) or (ii) always in the nucleus of target cells (for type II receptors). A second effect of ligand binding on several type I receptors, notably AR, GR and PR, is the dissociation of several chaperone proteins which maintain the receptor in a conformation optimal for hormone binding. Subsequent apposition of the hormone–receptor complex to target sequences in the promoters of target genes enhances their expression [1]. Such a functionally based model does not convey, however, the complexity of steps preceding initiation of transcription and recent research has begun to tease out the complexity of molecular events pursuant to ligand binding. It is now apparent that, by means of interaction with transcriptional coactivators, liganded receptor directs disruption of the nucleosomal structure around target genes by means of recruited chromatin-modifying enzyme activities. In addition, recruitment of basal transcription factors by coactivators is thought to lead to the establishment of a stable preinitiation complex at the hormone-regulated promoter. In this process, DNA-bound receptors and coregulators appear to function as large multiprotein complexes which, depending upon the cell/promoter context and stage of transcription, are thought to have multiple possible configurations.

3. Coactivators

3.1. Transcription-mediating proteins

In essence, the role of the activated nuclear receptor is to recruit and maintain a preinitiation complex at the promoter of the target gene. This is achieved by direct or indirect interaction of the liganded receptor with the basal transcription factors, a group of proteins comprised of RNA polymerase II, TATA-binding protein (TBP) and a host of TBP-associated proteins (TAF_{II}s), which are required for efficient regulated transcription of most eukaryotic genes [2]. In the 1980s, functional analysis of receptors had identified autonomous activation functions (AFs) within the amino-terminal and carboxy-terminal domains of the receptors which were thought to be key elements in transactivation. Studies on cell specificity and transcriptional interference of these AFs [3,4] gave rise to the suggestion that certain limiting receptor-specific factors, termed transcription intermediary factors [TIFs], mediated the transcriptional activity of the receptors [5].

Various independent lines of evidence have indicated that liganded receptors are capable of directly contacting basal transcription factors. In particular, TAF_{II}s, which appear to be cell-specific in their expression patterns, interact with liganded receptor [6, 7]. Three seminal studies suggested, however, that non-TAF_{II} proteins were important targets of liganded receptor. Isolation of a 160-kDa estrogen receptor (ER)-associated protein (ERAP-160) which co-purified with ER in the presence of ligand [8] suggested that ER underwent interactions with specific protein complexes prior to transcriptional initiation. Cavailles *et al.* (1994) published similar findings with respect to the receptor interacting proteins RIP-160, RIP-140 and RIP-80. The characterization of a 170 kDa GR-associated protein (GRIP-170), an enriched fraction of which stimulated GR transactivation *in vitro*, suggested that these endogenous cofactors were functionally limiting [9]. Notably, antihormones uncoupled the interaction of receptor with these proteins (collectively termed 'p160' proteins), suggesting that transcriptional activation was contingent upon the interaction of the receptor with these proteins. However, when cotransfected with receptor in a reporter assay, RIP-140 was not capable of significant coactivation of ER [10] and the function of the ERAPs and RIPs remains open to debate [11]. Several other receptor interacting proteins, such as the bromo domain-containing TIFs [12], Trip-1, the human homolog of the yeast transcriptional mediator Sug1 [13] and other Trips (thyroid receptor-interacting proteins), were identified and partially characterized, but their role in transcriptional regulation is, to date, largely undefined.

3.2. The SRC-1 subclass: cloning and functional domains

Using a genetic screen in a yeast-based human cDNA library to isolate PR ligand-binding domain-interacting proteins, our laboratory cloned and characterized a 160-kDa protein, steroid receptor-coactivator-1 (SRC-1), which bore no homology to RIP-140, TIF-1 or Trip-1. SRC-1 exhibits several properties which defined it as a prototypical coactivator for nuclear receptors [14]. Firstly, coexpression of SRC-1 with a variety of nuclear receptors in a reporter assay enhanced transactivation (5–10-fold) by the receptors, suggesting that it constituted a common limiting factor for the receptor superfamily. Secondly, coexpression of SRC-1 with two distinct receptors reversed the squelching, or transcriptional interference between the receptors, which had originally indicated the existence of limiting transcriptional intermediates [3, 4]. Thirdly, competitive coexpression of the receptor-interacting domain of SRC-1 (SRC-1 0.8) with SRC-1 in receptor cotransfection assays resulted in dominant-negative inhibition by SRC-1 0.8 of transactivation. Fourthly, SRC-1 contains two autonomous, transferable activation domains [15] which, when fused to heterologous DNA-binding domains, enhanced the expression of genes linked to heterologous *cis*-acting elements. Finally, addition of the antagonist RU486 abrogates the ability of SRC-1 to interact with and coactivate PR [14], suggesting a mechanistic basis for the antagonistic properties of RU486.

SRC-1, also designated NCoA-1 [16], given its molecular size, its widespread expression and functional interaction with a wide variety of nuclear receptors, was a plausible candidate for the biochemically-defined 'p160'. However, the subsequent cloning of GRIP1/TIF2/SRC-2[1] [17] and p/CIP [18] (also designated ACTR/RAC3/AIB-1/TRAM-1/SRC-3 herein) suggested that the term 'p160' encompassed a novel family of structurally-related nuclear receptor coactivators, the SRC-1 family. SRC-1, GRIP-1/TIF2/SRC-2 and p/CIP/SRC-3 exhibit common properties in the transcriptional activation of a wide variety of nuclear receptors [14, 16, 17, 19–25]. This family has a number of structural features in common, one of the most interesting being the presence of in their N-termini of domains present in the PAS (for Per-Arnt Sim homology)/bHLH (for basic helix–loop-helix) family of transcription factors. Members of the bHLH family are involved in regulation of cell type differentiation and proliferation and are characterized by the formation of homo- or heterodimeric complexes with bHLH partners for their function (for a review see Ref. [26]). Like other PAS-bHLH proteins [27], SRC-1 and TIF2 appear to be capable of forming multimeric complexes *in vivo* [28], but the role of the PAS domain in this interaction, if any, is unclear.

3.3. The LXXLL motif: interactions of coactivators with receptors

A second common structural feature of the SRC-1 family is the LXXLL sequence, a recurrent pentapeptide motif which appears to direct the interaction of SRC-1 family members (and other coregulators, see below) with their receptor partners [21, 29]. In order to encourage consensus, this review will adopt the proposed nomenclature [30] in discussion of these motifs. Heery *et al.* [29] defined the LXXLL motif as a crucial element in the interaction of RIP-140 and SRC-1 with nuclear receptors and showed that mutation of any of

[1] In the interests of brevity, we employ the unified nomenclature proposed by Li and Chen [92] who suggested the name 'SRC family' for this new family of coactivators. The name SRC-1 refers to SRC-1 NCoA-1, SRC-2 will refer to TIF2/GRIP-1/NCoA-2 and SRC-3 will refer to RAC3/AIB1/ACTR/TRAM-1/pCIP/NCoA-3.

the core LXXLL residues in these coactivators was sufficient to abrogate binding to nuclear receptors. Furthermore, they demonstrated the reciprocal importance for receptor–coregulator interaction of hydrophobic residues in the conserved helix 12 of the ER, a region corresponding to the functionally-defined AF-2 domain of nuclear receptors. Substantiating these results, Feng *et al.* [31] used scanning mutagenesis studies along with X-ray crystallography to depict the formation of a single site in AF-2 of the human TR which binds SRC-1 and GRIP-1/TIF2. Ligand binding induced the convergence of a small series of residues around the surface of a hydrophobic cleft in C-terminus of the TR by folding a C-terminal α-helix. These residues lie within portions of the TR that are conserved between nuclear receptors, suggesting that formation of such a groove might be a phenomenon which accompanies ligand binding by other members of the family [31]. Ding *et al.* [30] have gone some way towards explaining the existence of multiple SRC-1 family members by showing that receptors exhibited preferential binding to different SRC family members. AR, for example, binds preferentially to GRIP-1 over SRC-1. In addition, LXXLL motifs within a given coregulator exhibit binding specificity: the central domain of SRC-1 (NR boxes I–III) preferentially binds the LBDs of ER, PR, VDR and TR, while the more C-terminal NR-box IV strongly binds AR and GR.

3.4. Cointegrators: the CBP/p300 class of coactivators

CBP and p300 are ubiquitous, evolutionarily conserved proteins which have been shown to act as transcriptional coactivators for a host of diverse transcription factors, including CREB (cAMP-response element-binding protein) [32], STAT-2 [33] and p53 [34,35]. Moreover, CBP has been shown to exist in a stable preformed complex with RNA Pol II [36], suggesting that interaction of transcription factors with CBP, either directly or indirectly, might result in a direct link to basal transcription factors. It has been proposed [16,37] that nuclear receptors might also require the mediation of CBP p300 for efficient transactivation. It was shown that CBP interacted weakly with nuclear receptors in a ligand-dependent manner, enhanced RAR-mediated transactivation, and was capable of binding SRC-1 directly. p300/CBP are proposed to be limiting, common cointegrators for distinct but convergent signalling pathways, functioning to integrate multiple afferent signals into an appropriate response at a common promoter [16]. Results from our laboratory substantiate the role of CBP in steroid receptor signalling, indicating that CBP and SRC-1 synergistically activate transcription from ER and PR-regulated promoters [38]. Biochemical analysis suggests however that CBP and SRC-1 exist in largely distinct preformed complexes [28], and it may be that they interact only transiently when recruited by liganded receptor at the promoter.

3.5. Nuclear receptors and chromatin

In order for efficient spatiotemporal patterns of gene expression to occur *in vivo*, eukaryotic genes are required to exist in conformations which maximize access to the *cis*-acting elements with which DNA-binding transcription factors specifically interact, while minimizing basal, unregulated expression of these genes. The consequence of this requirement is the organization of eukaryotic genes into structurally-repressed nucleosomes, the integrity of which is dependent upon periodic arrays of DNA-binding histones. Nucleosomes are the basic repeating unit of chromatin and it is their malleable and plastic nature which permits the strictly regulated access of transcriptional proteins to key regions of genes, allowing finely regulated control of transcription of these genes [39]. Reduction of their net positive charge and affinity for DNA by acetylation of core histones has long been known to be an important preface to transcriptional activity *in vivo*, and numerous studies have shown that histones in regions of transcriptionally active chromatin are hyperacetylated [40]. The link between chromatin disruption and transcriptional activity is now well established: for example, a critical transcriptional adaptor in yeast, GCN5, was identified as histone acetyltransferase, an enzyme which catalyzes the transfer of acetyl groups to nucleosomal histones [41].

The discovery that p300, CBP and a p300/CBP associated factor (PCAF) all contained histone acetyltransferase activity [42–44] indicated that nuclear receptors might function in part by recruiting these proteins and directing nucleosomal modification at their target promoters. This notion was further strengthened by the identification of similar activity in the SRC family members SRC-1 and ACTR/SRC-3 [23,45]. Experimental evidence suggests that complexes containing these coregulators are recruited by receptor at hormone-regulated promoters [46], but the reason for the requirement of so many HATs is unclear. We are currently examining the possibility that different coregulator complexes may have different target histone specificities.

3.6. Chromatin modification: the SWI/SNF proteins

Studies in *S. cerevisiae* have provided convenient but far-reaching insights into the effect of transcriptional regulators on chromatin structure *in vivo*. Along with the ADA proteins, one of the first groups of yeast proteins to be identified and characterized as important transcriptional intermediaries were the SWI/SNF pro-

teins, which form a stable, preformed complex of approximately 2 MDa in size [47,48]. Subsequent studies have demonstrated that purified SWI/SNF complexes have intrinsic ATPase activity and function, at least in part, by coupling ATP hydrolysis to nucleosomal remodelling at diverse promoters to facilitate the interaction of basal transcription factors with these promoters [49]. Unlike HATs, SWI/SNF complexes do not carry out covalent modification of histones, but rather catalyze the uncoupling of ionic interactions between histones and their substrate DNA. It is now clear that the SWI/SNF complex is highly conserved evolutionarily and SWI/SNF complexes have since been purified and characterized from mammalian sources [50]. Human SWI/SNF homologs have been found to enhance the activation functions of GR, ER and RAR [51–53], and it has been shown that GR directs ligand-dependent nucleosomal remodeling activity of the SWI/SNF complex in yeast [54]. The fact that SWI/SNF proteins and nuclear receptors interact functionally in yeast systems is strong evidence of their potential role in nuclear receptor signalling *in vivo*. One of the most highly conserved members of the SWI/SNF complex is SWI2/SNF2, which contains ATPase activity [55] and which is encoded by the *swi*2 and *snf*2 genes. Two closely-related mammalian homologs of the yeast *swi2/snf2* genes are termed *brahma* and *brahma*-related gene-1 (*brg*-1). BRG-1 (hSNF2β), the product of the *brg*-1 gene, has been shown to interact with GR in a ligand-dependent manner [56]. Moreover, BRG-1 enhances transcriptional activation by ER in transient transfections in mammalian cells [57], further suggesting that mammalian SWI/SNF proteins may be key elements in nuclear receptor action. BRG-1 has been proposed to exist in a stable complex with SRC-1 [57] and our own data show that a minor pool of BRG-1 copurifies with SRC-1 [28].

3.7. E3 ubiquitin-protein ligases

Included in a fourth subclass of coactivators, which reiterates the role of enzymatic activities in transcriptional regulation by nuclear receptors, are the E3 ubiquitin-protein ligases RPF-1 [58] and E6-AP (Nawaz *et al.*, in press). This subclass of coregulators differs from the SRC-1 family, p300/CBP cointegrators and SWI/SNF homologs, in that they contain ubiquitin-protein ligase activity rather than HAT activity. These proteins were initially identified as factors required for defining substrate specificity in proteolytic degradation by the proteosome system. E6-AP does not require its ubiquitin protein-ligase function for coactivation of nuclear receptors, indicating that it has separable functions as a coactivator and a ubiquitin-protein ligase. Our laboratory and others are currently attempting to define the functional basis of coactivation by E6-AP and RPF-1. We have recently established that mammalian E6-AP and RPF-1 copurify precisely by gel filtration, suggesting their possible existence in a common complex, and that these ubiquitin-protein ligase coactivators synergistically enhance transcriptional activation by PR [28].

3.8. Coactivator interactions: modular complexes for a multistep process

Much of the discussion of coregulators has referred to the formation of transcriptional complexes, in which multiple receptor–coregulator and coregulator–coregulator interactions are envisaged to occur. Individual studies have documented many such interactions: liganded nuclear receptors interact with the SRC-1 family members SRC-1/NCoA-1 [14,16,28], GRIP-1/TIF2/SRC-2 [17,19,20,28] and p/CIP/RAC3/AIB-1/ACTR/TRAM-1/SRC-3 [21–25], the cointegrators CBP and p300 [38], PCAF [46,59], human homologs of the yeast SWI/SNF proteins [56] as well as the E3 ubiquitin-protein ligase family members RPF-1 [58] and E6-AP (Nawaz *et al.*, in press). In addition, multiple coregulator/coregulator interactions have been proposed, including p/CIP/CBP [21], CBP/PCAF [44], SRC-1/CBP [16], SRC-1/p300 [60], SRC-1/PCAF [45] and SRC-1/BRG-1 [57].

While these studies provide valuable information on potential interacting partners for coregulators, they yield little comparative data, such as the relative strength and importance of these interactions in the context of multiprotein complexes. Pertinent questions are raised concerning the functional organization of coactivators: do they exist in preformed complexes, or are transcriptional complexes assembled *de novo* by activated receptor? On the one hand, the free energy required to assemble large, transient complexes from individual components is intuitively prohibitive. On the other, the existence of large readily-primed complexes increases the potential for unregulated transcription. Our own comparative analysis of coregulator interactions indicates that they are governed by a hierarchy *in vivo*, resulting in largely distinct subcomplexes of different types of coregulators [28]. For example, minor pools of p300, BRG-1 and PCAF copurify with the elution peak of SRC-1 [28], supporting the contention that these proteins may exist in preformed complexes with SRC-1 [45,57,60]. In contrast, proposed complexes such as SRC-1/CBP [16] do not appear to exist in the steady state under our conditions. In agreement with our own data, Korzus *et al.* [61] have shown that promoters coupled to different enhancer elements (RARE, CREB and STAT) require different combinations of coactivators for maximal coactivation at each promoter. It may be that this is a functional consequence of the evidently modular character of

coactivator complexes, a character which we would predict contributes to cell and promoter-specificity of transcriptionally active complexes.

Transcriptional regulation is known to be comprised of multiple, functionally distinct steps, including chromatin remodelling and transcriptional activation. To illustrate this distinction, GR which failed to recruit BRG-1-containing complexes was capable of activating transcription from naked transiently transfected templates, but not from stably integrated templates [56]. Moreover, the elegant oocyte reconstitution system of Wong *et al.* [62] has shown that chromatin disruption is insufficient for activation of the TRβ-gene. In addition, Kraus and Kadonaga [63] have used an *in vitro* transcription system to draw a clear line between the role of p300 in 'preinitiation' and 'reinitiation' complexes assembled by liganded ER: from their data, it appears that the functional requirement for p300 is reduced for subsequent rounds of transcription after the first round.

The functional evidence for the multistep nature of transcription has been supported by biochemical studies in respect of TR [64,65]. Constitutively liganded tagged TR copurified from HeLa cell nuclear extract with a series of proteins ranging in size from 80-220 kDa in size. When an *in vitro* TR transcription system was supplemented with purified fractions containing these proteins, coactivation was modestly enhanced over basal levels [65]. Interestingly, none of the TRAPs represented previously-identified coregulators. Further characterization of one of the members of this complex identified a protein, TRAP-220, which contained a consensus LXXLL motif and interacted with TR directly [64]. Similar complexes have been observed for VDR [66] and ER [67], the latter containing a protein with casein kinase 2-like activity. A model has been envisaged for the role of TRAPs in receptor transactivation, envisaging initial binding of complexes containing SRC-1 family members, CBP p300, PCAF and other complexes. Displacement of these initial coactivator–coregulator complexes by TRAP-like complexes may then occur subsequently and these complexes may assume a more important role in repetitive initiation of transcription.

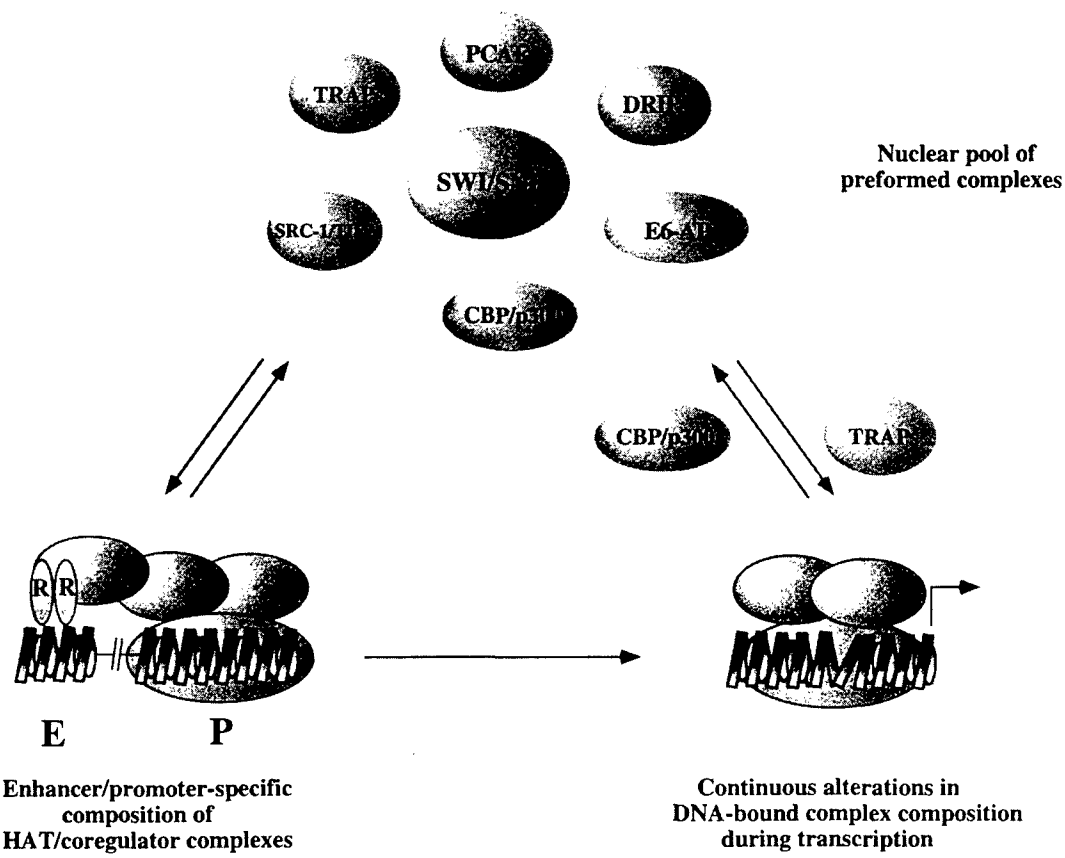

Fig. 2. Dynamic model of the potential role of preformed coregulator complexes in nuclear receptor (R) action. A nuclear pool of coregulators assembled into preformed complexes allows for enhancer (E) and promoter (P) specificity in transcriptional initiation and a rapid assembly of the preinitiation complex. In addition, the different functional stages of transcription are likely to require multiple complex configurations and there is likely to be continuous exchange between the nuclear pool and the transcriptional complex.

3.9. A model for coactivator action

These diverse lines of evidence may be incorporated into a model of coregulator action (Fig. 2) in which multiple preformed complexes, with specific functional characteristics, are recruited by liganded receptor at different stages during transcriptional activation. The functional diversity arising from such an arrangement facilitates the simultaneous assembly and disassembly of transcriptional complexes at diverse promoters, allowing the efficient and balanced integration of signals by different transcriptional activators. Mechanistically, the model concurs with data from our own and other laboratories and is in keeping with the notion of transcription as a modular process, requiring the mediation of functionally different subsets of complexes at different points. Biochemically, the complexes are largely distinct, but significant overlap exists between some of them, symptomatic of heterogeneity of their composition. Although the model conveys a static situation, we envisage a spectrum of interactions, resulting in fluid compositions of transcriptional complexes.

4. Corepressors

Along with punctual activation of target genes in response to hormones, an efficient spatiotemporal pattern of gene regulation by nuclear receptors requires timely silencing of these genes in the absence of ligand. While this review has concentrated on coactivators as transcriptional intermediaries for nuclear receptors, mechanistically symmetrical models are emerging for the role of corepressor proteins in transcriptional silencing by nuclear receptors. Similar to its role in gene activation, chromatin is an intrinsic part of the mechanism by which nuclear receptors silence their cognate promoters. Functional data suggests that, as in the case of transcriptional activation, multiple factors are recruited by unliganded receptors to participate in gene silencing. Moreover, evidence exists to suggest that these factors function as components of large multiprotein complexes which co-ordinate target promoter silencing by nuclear receptors.

The existence of repression domains in nuclear receptors was first suggested by experiments which indicated that transferable, autonomous silencing domains mediated transcriptional silencing by TR and PR [68,69]. Soluble corepressor proteins were postulated to exist in cells and to interact with receptor repressor regions [70]. Since these initial studies, several proteins have been identified as potential mediators of repression by nuclear receptors corepressor complexes. RIP13/NCoR [71–73] and SMRT [74] were cloned as molecules which interacted in a ligand-dependent manner with RXR and TR and which contained transferable domains capable of silencing transcription at heterologous promoters. These corepressors are now known to have a broad range of specificity within the nuclear receptor superfamily and have been proposed to mediate repression not only by type II receptors, but also by type I [75,76] and orphan [77–79] members of the family.

The transcriptional efficiency of certain genes in yeast is known to be regulated by several key proteins, including RPD-1, SIN3 and RPD-3. The discovery of homology between RPD-3 and a subunit of a yeast histone deacetylase complex [80] and the cloning of a mammalian homolog of RPD-3, histone deacetylase I [81], suggested that certain mechanisms of transcriptional repression might be conserved in eukaryotes. SIN3 was known as one of a family of proteins which contained transferable repression domains which mediated transcriptional repression in yeast [82–84]. The conservation of transcriptional mechanisms in yeast permits the reconstitution of steroid-receptor mediated transactivation in this system. Initial data from our laboratory identified SIN3 as a negative regulator of PR activity in yeast [85]. Numerous studies have since established functional interactions between NCoR/SMRT, histone deacetylases and the mammalian homologs of SIN3, mSin3a and mSin3b, in the context of transcriptional silencing by TR and RXR [86–89]. Moreover, biochemical characterization of histone deacetylase activity in *Xenopus* oocytes indicates that Sin3 proteins copurify with NcoR and HDAC-2 in multiprotein complexes [90,91]. An integrative model of repression by nuclear receptors envisages recruitment by unliganded receptors of large, preformed complexes containing corepressors and associated histone deacetylase activities.

5. Conclusions

Steroid, retinoid and thyroid hormones direct finely-regulated patterns of gene expression which coordinate processes involved in development, differentiation and reproduction. While these events are relatively well characterized on a physiological level, the molecular events through which these hormones and their receptors regulate the transcription of their target genes are only partly understood. It is now emerging that transcriptional control is a multistep process, a fact reflected in the diversity of the coregulators, and their intrinsic enzyme activities, which liganded receptor recruits to the promoter. These coregulators are organized into stable, preformed multiprotein complexes, the modular character of which may facilitate the efficient assembly of functionally diverse complexes by a single receptor dimer. In addition, the modular charac-

ter of these complexes provides the potential for different activators to assemble diverse configurations of regulatory complexes at their cognate *cis*-acting elements. It is anticipated that further study of nuclear receptor coregulators and their complexes will yield significant insights into the basis of the complexity of signalling by steroid, thyroid and retinoid hormones.

Acknowledgements

The authors would like to thank Barb Tykal for proofreading and Rainer Lanz, Mark Burcin and other members of the O'Malley laboratory for helpful discussions.

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22 Coactivators and Corepressors

Neil J. McKenna, Zafar Nawaz, Sophia Y. Tsai, and Ming-Jer Tsai

AFFILIATION: DEPARTMENT OF MOLECULAR AND CELLULAR BIOLOGY
BAYLOR COLLEGE OF MEDICINE
ONE BAYLOR PLAZA, HOUSTON TX 77030

CONTENTS

1. COACTIVATORS

1.1. Eukaryotic Transcription

At the vast majority of eukaryotic promoters, transcription is mediated by RNA polymerase II (RNA pol II). Activated transcription at these promoters is believed to require assembly of a transcriptional preinitiation complex, comprising general transcription factors (GTFs) such as TFIIB, TFIID, a multicomponent complex composed of TATA binding protein (TBP) and the TBP-associated factors (TAF_{II}s), as well as RNA pol II itself. In many cases, the net rate of assembly of such complexes is enhanced by transcription factors that bind specific recognition sequences within promoters. Functional interactions between activated transcription factors and general transcription factors are thought to be mediated by *coactivators*, which may be broadly defined as molecules recruited by transcription factors to enhance their transcriptional activity. Within this definition lies a wide range of functions that together are required for efficient activation by transcription factors.

This section briefly describes ~~several~~ classes of molecules that act as coactivators for members of the nuclear receptor superfamily. For a thorough review of these nuclear receptor coactivators the reader is referred to recent reviews (Horwitz et al., 1996 and McKenna et al., 1999). The nuclear receptor superfamily comprises functionally similar ligand-inducible transcription factors that regulate transcription of target genes in response to hormonal ligands. These molecules have a common structure, particularly in the case of conserved activation functions (AFs) in their C-termini (AF-2) and N-termini (AF-1). Upon binding of the receptor to specific DNA sequences (response elements) in the promoter of their target genes, these AFs are believed to initiate a sequence of interactions that results in assembly and stabilization of GTFs at the target promoter.

1.2. Evidence for the Existence of Coactivators

Although a number of direct interactions between nuclear receptors and GTFs have been reported, such as those between TFIIB and thyroid hormone receptor (TR), TBP and retinoid X receptor (RXR) AF-2, estrogen receptor (ER) and TBP, and TFIIB and vitamin D receptor (VDR), abundant historical evidence has suggested that receptors recruit specific non-GTF targets after ligand binding. Squelching studies, which identified interference between receptors in transient cotransfection assays, implied that receptors competed for essential cofactors, the levels of which limited the transcriptional potential of the competing pools of receptor in these assays. The identification

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Table 1
Selected Nuclear Receptor Coactivators

Cofactor	Alternative Names	Function: Activation Domains	Function: Acetylation	Function: Ubiquitin Pathway	Function: ATPase	Reference
SRC-1 family (Section 1.3.)						
SRC-1	NCoA-1, ERAP-160	√	√			Onate et al., 1995 Xu et al. 1998 and references therein
SRC-2	TIF2 NCoA-2	√				Voegel et al., 1998 and references therein
SRC-3	p/CIP, ACTR, AIB-1, TRAM-1	√	√			Li and Chen, 1998 and references therein
Cointegrators						
CPB		√	√			
p300		√	√			*See* Chapter by _____, this volume
Others (Section 1.4.)						
$TAF_{II}s$		√				
TRAPs/DRIPs			√			Rachez et al., 1998 and references therein
Trip-1	Sug-1			√		*See* references in McKenna et al., 1999
E6-AP		✓		√		McKenna et al., 1998 and references therein;
RPF-1				√		Nawaz et al., 1999 and references therein
BRG-1	SWI2/SNF2				√	*See* references in McKenna et al., 1999

of liganded estrogen receptor-interacting proteins such as the ERAPs and RIPs reinforced the supposition that specific downstream targets, distinct from GTFs, are contacted by activated receptors.

1.3. The SRC Family

1.3.1. SRC-1/NCoA-1

In 1995, our laboratory described the first cloning and characterization of a common transcriptional coactivator for nuclear receptors, steroid receptor coactivator-1 (SRC-1), a prototype for an emerging family of nuclear receptor coactivators, the SRC family (Onate et al., 1995; Table 1). This structurally and functionally redundant family consists of three members: SRC-1 (NCoA-1), SRC-2 (GRIP-1/TIF2; *see* Section 1.3.2.), and SRC-3 (p/CIP/ACTR/RAC3/AIB-1/TRAM-1; *see* Section 1.3.3.).

SRC-1 interacts with and enhances the ligand-dependent transactivation of a broad range of nuclear receptors, including PR, GR, ER, TR, and RXR. Importantly, SRC-1 partially reverses the squelching of PR transactivation by cotransfected ER, indicating that it can relieve the titration of a common, limiting factor recruited in vivo by the AF-2s of ER and PR for efficient transactivation. In addition, SRC-1 contains two autonomous, transferable activation domains that are capable of stimulating transcription when fused to the DNA binding domain of the yeast transcription factor GAL4. Sequence analysis of the amino terminal region of mSRC-1/NCoA-1 has identified bHLH (for basic helix–loop–helix) and PAS (for Per/Arnt/Sim) domains. The conservation in the SRC family of these domains, known to mediate functional interactions between proteins containing these domains, indicates that SRC family members might extend the regulatory compass of nuclear receptors to signaling pathways mediated by other bHLH/PAS factors. In addition,

sequence analysis of SRC-1 and other SRC family members has indicated their conservation of short LXXLL motifs, or NR boxes, which appear to contribute to the specificity of coactivator–receptor interactions (Heery et al., 1997). Targeted deletion of SRC-1 in mice confirms the in vivo function of SRC-1 as a coactivator for nuclear receptors, as well as the biological role of redundancy within the SRC family. Although both sexes are viable and fertile, the response of steroid target tissues to hormonal stimulation is significantly reduced in the SRC-1 null mutant, although overexpression of SRC-2/TIF2 (*see* below) appears to be a compensatory mechanism in certain tissues of the mutant (Xu et al., 1998).

1.3.2. SRC-2: GRIP-1/TIF2

Another member of the SRC family, transcription intermediary factor-2 (TIF2 or hSRC-2) and GR-interacting protein (GRIP-1 or mSRC-2) associates in a ligand-dependent manner with receptor hormone binding domains and enhances their transcriptional potential. Like SRC-1, TIF2/GRIP-1 contains defined activation domains capable of stimulating transcription when tethered to a heterologous DNA binding domain. In addition, TIF2, like SRC-1, is capable of relieving transcriptional squelching by ER (Voegel et al., 1998 and references therein).

1.3.3. SRC-3: p/CIP/RAC3/ACTR/AIB-1/TRAM-1

In terms of sequence identity, the most highly variable member of the SRC family is a polymorphic protein referred to as p/CIP (p300/CBP cointegrator-associated protein), ACTR, RAC-3 (receptor-associated coactivator 3), AIB-1 (amplified in breast cancer-1), TRAM-1 (thyroid receptor activator molecule), and SRC-3 (Li and Chen, 1998 and references therein). Li and Chen (1998) have proposed that the inclusive name of SRC-3 be adopted for this protein. In parallel with SRC-1 and TIF2/GRIP1, SRC-3 interacts with and coactivates a wide variety of nuclear receptors in a ligand-dependent manner, including RAR, TR, RXR, GR, ER, and PR. The p/CIP isoform, however, exhibits markedly different specificity from other SRC family members. It fails to coactivate RAR-mediated transcriptional activation, but enhances the transcriptional activity of a number of different activators, including STAT5 and cAMP response element binding protein (CREB), factors previously shown to be primarily dependent upon the transcriptional cointegrator CREB-binding protein (CBP) for efficient activation (*see* Chapter 23). This variable specificity might reflect the considerable sequence divergence between p/CIP and other SRC-3 isoforms, which exhibit only minor sequence variations (Li and Chen, 1998 and references therein).

1.4. Other Coactivators

1.4.1. E6-AP/RPF-1

The identification by our laboratory and others of the E3 ubiquitin–protein ligases RPF-1 and E6-AP as coactivators for nuclear receptors has implicated protein degradation as an important component of transcriptional activation. E3 ubiquitin–protein ligases target proteins for degradation by the ubiquitin pathway. E6-AP is recruited by and enhances activation by receptors such as PR, ER, and AR in a ligand-dependent manner. In addition, E6-AP partially reverses squelching between ER and PR and contains an intrinsic activation function in its N-terminal domain. Interestingly, our laboratory has recently shown that E6-AP and RPF-1 synergistically enhance PR transactivation, and that they may exist in a common complex in vivo (McKenna et al., 1998; Nawaz et al., 1999 and references therein).

1.4.2. Trip-1/sug1

The requirement of targeted protein degradation for efficient activation by nuclear receptors is further implied by the identification of Trip-1, a protein that interacts with TR and RXR baits in a yeast two-hybrid assay in a ligand-dependent manner. A member of the CAD (conserved ATPase domains) family of proteins, Trip-1 exhibits considerable sequence similarity with the yeast transcriptional mediator Sug1, a factor required for suppression of a mutation in the transcriptional activation domain of GAL4. Although originally suggested to be a component of the Pol II holoenzyme complex, the existence of Sug-1 in the 2MDa yeast 26S proteosome complex has been reported, and has been correlated with reduced ubiquitin-dependent proteolysis in *sug1* mutants. While the precise role of protein degradation in nuclear receptor transactivation is currently unclear, it is widely thought that activated receptors undergo functionally distinct interactions with multiple protein complexes. It may well be that timely recruitment of protein-degrading pathways to the promoter processes one complex(es), allowing the receptor to interact with another complex(es) during the next phase of transcription. Alternatively, ubiquitin pathways may be required to clear liganded receptor itself from the promoter (*see* references in McKenna et al., 1999).

1.4.3. TRAPs/DRIPs

By adopting a biochemical approach to the identification of nuclear receptor coactivators, two groups have identified multiprotein complexes that interact with liganded TR (TRAPs) and VDR (DRIPs). When added to TR- and VDR-dependent in vitro transcription systems, purified TRAPs and DRIPs enhanced the transcriptional activity of their respective receptors. Although the exact nature and function of these complexes is unknown, they have been shown to contain no previously identified coactivators such as SRC family members, CBP, ERAP140, RIP140, and GTFs. A model has been proposed that such complexes may mediate repetitive rounds of transcription mediated by TRα and VDR. Initial recruitment of complexes containing the cointegrator CBP and members of the SRC family (*see* Section 1.3.1.) would be followed by displacement of these complexes (a step conceivably mediated by ubiquitin pathway-linked proteases) and interaction of the receptor with TRAP/DRIP-like complexes (Rachez et al., 1998 and references therein).

1.5. Chromatin Modification by Coactivators

The default state of many genes is thought to be one in which transcriptional activity is repressed by local chromatin structure, which acts to block access of *trans*-acting factors to the promoter. Core histones are thought to be central to the cohesion of this repressive state, and their abundance of positively charged lysine side chains have been suggested to interact with the negatively charged phosphate backbone of the DNA double helix. Acetylation of core histones is known to reduce their affinity for DNA by reducing their net positive charge and weakening their interaction with DNA. Since the identification of the yeast transcriptional adaptor GCN5 and its human homolog PCAF as proteins that catalyze the transfer of acetyl groups to histone lysine side chains (histone acetyltransferases or HATs), it has become clear that many nuclear receptor coactivators possess this activity. The SRC family members SRC-1 and SRC-3/ACTR, the cointegrators p300 and CBP, the p300/CBP-associated factor PCAF, and at least one member of the DRIP complex contains HAT activity. It seems, however, that acetylation by coactivators is not limited to histones: DNA binding by the transcription factors p53 and GATA-1 is stimulated after their acetylation by p300, and the DNA binding domain of the PR appears to be a target for acetylation by PCAF (M. Burcin, *personal communication*).

In addition to targeting HATs to DNA, evidence suggests that nuclear receptors also recruit protein complexes involved in the manipulation of chromatin domains to favor transcriptional activation. Members of the SWI/SNF complex, which couples ATP hydrolysis to noncovalent chromatin remodeling, historically have been associated with transactivation by nuclear receptors, and recent data have elucidated the molecular basis of this association. Mutations in SWI protein-encoding genes prevent transactivation in yeast of a GR-responsive reporter gene in the presence of cotransfected GR, whereas a wild-type yeast (stain) was able to support GR-dependent transactivation. Further, a human homolog of the SWI2/SNF2 proteins, BRG-1, interacts with ER in a ligand-dependent manner in a yeast two-hybrid assay, and the nucleosomal remodeling activity of the SWI/SNF complex is required for GR function in yeast. To substantiate this in a mammalian context, we have shown that GR regulation of a stably integrated MMTV promoter is dependent upon recruitment of complexes containing BRG-1 (see references in McKenna et al., 1999).

The nuclear receptor-interacting proteins TIF-1α and TIF-1β have been implicated by association in processes of chromatin remodeling. TIF-1α interacts with the heterochromatin-associated proteins mHP1α, MOD1 (HP1β), and MOD2 (HP1γ) which in turn interact with mSNF2-β, a member of the SWI/SNF chromatin-remodeling complex. A model has been suggested for TIF-1s in transcriptional regulation, in which formation of transcriptionally inactive heterochromatin by TIF-1s effects repression, and ligand-dependent association of TIF-1s with receptors mediates euchromatin formation (Le Douarin et al., 1998 and references therein) (*see also* Section 2.2.1.3.).

1.6. Transcriptional Mediation by Coactivators

Although chromatin modification is the most thoroughly characterized process in the cascade of events that likely accompany transcriptional coactivation by nuclear receptor coactivators, another fundamental component is their mediator function, which refers to their ability to direct interactions between activated receptor, GTFs and, ultimately, RNA pol II. Their possession of multiple autonomous activation domains and receptor interaction motifs suggests that they occupy a pivotal position in the assembly of the final preinitiation complexes at transcriptionally active promoters. To support this assertion, SRC-1 has been shown to interact directly with a number of basal transcription factors including TBP and TFIIB. Our

laboratory has shown that on naked DNA, a dominant-negative form of SRC-1 specifically titrates PR-mediated reporter gene activity in an in vitro transcription assay, suggesting that chromatin disassembly is insufficient *per se* for PR target gene activation. A two-step model for transcriptional induction by steroid receptors, suggests that chromatin modification by SRC-1, PCAF, and other coactivators (step 1) accompanies their mediation of functional interactions between activated receptor and GTFs (step 2) during efficient transcriptional activation (Jenster et al., 1997 and references therein).

1.7. Coactivators in Disease States

Since their initial characterization, several nuclear receptor coactivators have been implicated in disease states. AIB-1/hSRC-3 is consistently overexpressed in a high percentage of primary breast tumors and cultured breast cancer cell lines, often against a background of relatively low expression levels of SRC-1 and SRC-2/TIF2. These results indicate that AIB-1/hSRC-3 is involved in breast tumorigenesis, and that the selective advantage that this overexpression evidently affords is not constrained by the comparatively low levels in these tumors of SRC-1 and TIF2. There are also instances where levels of coactivator are normal, but their affinity for a specific receptor is reduced, as is the case with the TR in patients with generalized resistance to thyroid hormone (GRTH). A variety of TR mutations in GRTH patients are now known to affect the interaction of these TR mutants with coactivators such as SRC-1. A TR AF-2 mutant, E457D, which bound hormone normally and recruited NCoR in the absence of hormone, failed to bind SRC-1 in the presence of hormone, and was a strong dominant negative inhibitor of wild-type TR transactivation. In addition, it has been shown that although a GRTH TR mutant, T277A, containing a mutation in the ligand binding cavity bound ligand normally, it exhibits impaired recruitment of SRC-1 and SRC-3/ACTR in comparison with wild-type TR.

1.8. Nuclear Receptor Coactivators: A Working Model

The plethora of nuclear receptor coactivators identified in the literature to date should not necessarily be taken to imply that they act simultaneously with the activated receptor in a single large complex. Chromatin modification and contact with GTFs (mediation) (Sections 1.5. and 1.6.) are only two of many functions that coactivators undertake for efficient transcriptional regulation by nuclear receptors. In addition, evidence suggests that promoter-specific patterns of coactivator recruitment govern expression of specific gene networks in vivo. To this end, coactivators appear to exist, in the steady state at least, in smaller subcomplexes, which likely undergo combinatorial association into higher order, possibly promoter specific comformations. Indeed, liganded receptor has been shown to interact with complexes that contain none of the more familiar coactivators. Within this model, there may be hierarchical interactions, and evidence suggests that the interactions of liganded receptor with members of the SRC-1 family may be relatively stable (McKenna et al., 1998), implying that such complexes may be important intermediates during transcriptional activation. The role of protein-processing complexes recruited by coactivators such as E6-AP conceivably involves enabling a single receptor dimer to interact with multiple protein complexes over a given time period (Fig. 1). The recruitment of these and other coactivator enzyme activities by nuclear receptors during transcriptional activation highlights the multifunctional nature of this process (Korzus et al., 1998; McKenna et al., 1998 and references therein). Such complexity is reiterated in the functions of another class of proteins recruited by nuclear receptors for efficient regulation of transcription, the corepressors.

2. COREPRESSORS

2.1. Introduction

2.1.1. Transcriptional Repression

Like activation, transcriptional repression also plays an important role in normal cellular Members of a nonsteroid subfamily of the nuclear receptor superfamily, such as thyroid hormone receptor (TR), retinoid acid receptor (RAR), and vitamin D receptor (VDR) as well as many orphan receptors are known to be involved in transcriptional repression. These receptors have the ability to repress the transcription of their target genes in the absence of hormones. However, in the presence of hormone, they activate the transcription of their target genes.

Gene repression by nuclear hormone receptors appears to involve different mechanisms. In the first case, steric hindrance, the simplest mechanism of repression, involves blocking the access of transcription factors to the promoter by unliganded receptors. Second, interference and/or sequestration involves specific protein–protein contacts between an unliganded receptor and the components of the transcription initiation complex. Third, unliganded receptor

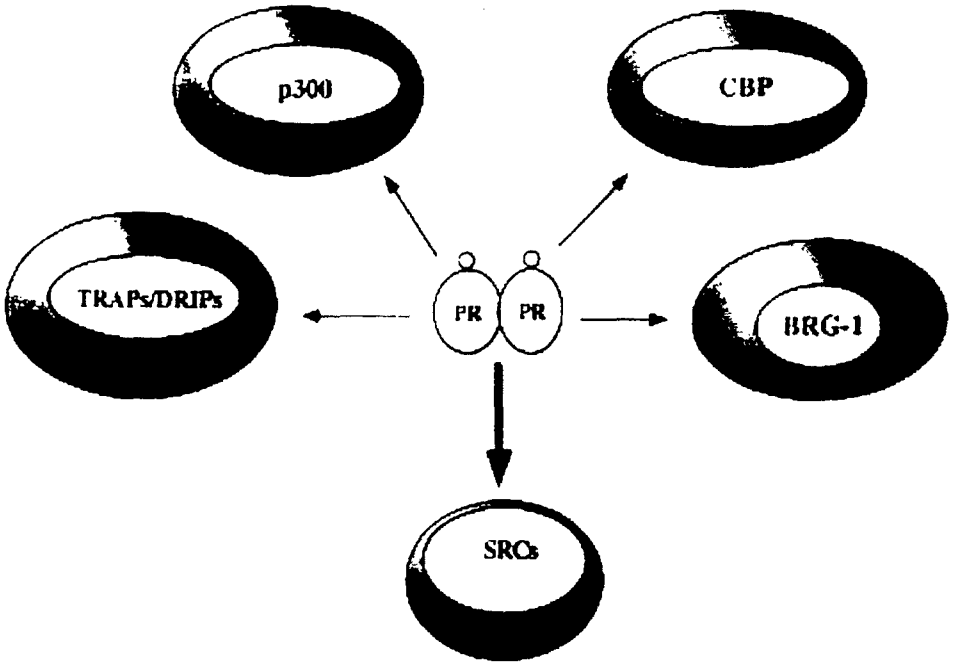

Fig. 1. Interaction of activated receptor with multiple coactivator complexes during transcriptional regulation. The association of SRC family members with liganded PR appears to be relatively strong and may be an important intermediate in PR transactivation. (Adapted from McKenna et al., 1998.)

recruits corepressors, enzymes and other proteins to the promoter region of target genes, thereby altering the acetylation status of histones and establishing transcriptionally inert chromatin.

2.1.2. Evidence for the Existence of Corepressors

The repressor function of nuclear hormone receptors has been localized to their ligand binding domains (LBD), and this function of the receptors is transferable to a heterologous DNA binding domain. This suggests that these receptors contain active repressor domains. Nuclear receptors may mediate their repressor function in part through the basal transcription factor, TFIIB. The supporting evidence for this comes from the fact that the LBD of TR interacts with the N-terminal region of TFIIB (Baniahmad et al., 1995). This interaction is hormone sensitive, suggesting that it is important for TR repressor function. The LBD of TR can be divided into two halves, each of which has no repressor activity by itself. However, when expressed together, they can work in *trans* to elicit full repressor activity, suggesting that at least two target interactions are required for repressor function.

The repressor function of TR can be reduced by overexpression of either the LBD of TR or the v-*erb*A, a mutant form of TR that has lost the ability to bind to hormone and exhibits a strong constitutive repressor activity. Furthermore, a chimeric receptor containing amino acid residues 120–392 of chicken TR fused to the yeast GAL4 DNA binding domain and the transactivation domain of the herpes simplex virus VP16 does not activate transcription when expressed alone. However, this chimeric receptor is able to activate transcription when coexpressed with unliganded TR and RAR. These findings imply the existence of cellular corepressors that are necessary for efficient repression by nuclear receptors. The existence of corepressors is substantiated further by the identification of amino acids in receptors that are critical for both repressor function and corepressor interaction. Like nonsteroid receptors, evidence also indicates the existence of corepressors for steroid receptors such as progesterone (PR) and estrogen (ER) receptors. These receptors recruit corepressors when bound to antihormones, but it is unclear at present whether these receptors are associated with corepressors in the absence of hormone (Smith et al., 1997 and references therein).

In recent years, experiments using the yeast two-hybrid and biochemical screening assays have identi-

Table 2
Nuclear Receptor Corepressors and Other

Corepressor	*Homologs*	*Interacting Protein(s)*	*kDa*	*Section (Accession No.)*
Nuclear receptor corepressors				
N-CoR/RIP13	TRAC1, SMRT/ TRAC2	TR, RAR, VDR, PR, ReV-Erbs, RORα, COUP-TFs, DAX-1, PLZF, SAP30, SIN3, TAFII32, TAFII70, TFIIB, mSiah2	270	2.2.1.1. (U35312)
SMRT/TRAC2	N-CoR/TRAC1	TR, RAR, RXR, PPARα, PR, ER, RORα, v-*erb*A, COUP-TFs, PLZF, SIN3, LAZ3/BCL6, HDAC-2	168	2.2.1.2. (U37146)
TIF1α		RXR, RARα, VDR, PR, ER, HP1, MOD1	112	2.2.1.3. (X99644)
TIF1β	KAP-1	KOX1/ZNF10, HP1, MOD1, KRAB	92	2.2.1.3. (X97548)
SUN-CoR		TR, RevErb, SMRT, N-CoR	16	2.2.1.4. (AF031426)
NSD1		RAR, TR, RXR, ER	285	2.2.1.5. (AF064553)
SSN6/TUP1		yα-2MCM1	106	2.2.1.6. (283218)
SIN3		MaD-MaX, SAP30, RPD3/HDAC-1, PLZF	105	2.2.1.7. (2137222)
KRAB family corepressors				
KAP-1	TIF1β	KRAB, KOX1/ZNF10, HP1, MOD1	89	2.2.2.1. (013263)
KRIP-1	TIF1	KRAB	92	2.2.2.2. (U67303)
Universal corepressors				
DR1		E4BP4, TBP	19	2.2.3. (1928868)
DRAP1		DR1	21	2.2.3. (1244714)
Other corepressors				
NAB1		NGFIA/Egr, KROX20	63	2.2.4.1. (1197669)
NAB2		NGFIA/Egr, KROX20	58	2.2.4.1. (1206027)
Gro		Hairy, Engraled, Tcf/Lef	79	2.2.4.2. (121620)
HIPKs		NK-Homeoproteins	130–133	2.2.4.3. (AF077658-60)

fied and characterized several different corepressor proteins for a diverse group of transcription factors. Based on the class of transcription factors with which these corepressors interact, they can be classified into four different groups as shown in Table 2: nuclear hormone receptor corepressors, KRAB family corepressors, universal corepressors, and other corepressors.

2.2. Corepressor Classes

2.2.1. Nuclear Hormone Receptor Corepressors

Several different corepressor proteins for nuclear hormone receptors have been cloned and characterized, a list of which is shown in Table 2. For a more thorough review of nuclear receptor corepressors, see Horwitz et al. (1996) and McKenna et al. (1999).

2.2.1.1. N-CoR/RIP 13. *N*uclear receptor *co*repressor (N-CoR)/*R*XR *i*nteracting *p*rotein 13 (RIP 13), a 270-kDa protein, was originally identified as a retinoid X receptor (RXR), RAR, and TR interacting protein. N-CoR/RIP 13 interacts with the hinge region (CoR box) of the nuclear hormone receptors only in the absence of hormone. Addition of hormone results in the release of N-CoR/RIP 13 from receptors. NCoR also interacts with antihormone-bound steroid hormone receptors, orphan receptors, and several other diverse groups of proteins such as histone deacetylases and a protein, Siah2, that targets NCoR for degradation by proteosomal degradation pathway enzymes. Deletion analysis of N-CoR/RIP 13 has identified two receptor interacting domains (RIDs) in, the C-terminus and three silencing domains (SDs) in the N-terminus region of the protein (Horlein et al., 1995).

2.2.1.2. SMRT/TRAC2. The *s*ilencing *m*ediator for the *r*etinoid and *t*hyroid hormone receptor (SMRT)/*t*hyroid *r*eceptor *a*ssociated *c*ofactor 2 (TRAC2) was isolated from a human lymphocyte cDNA library. SMRT, a 168-kDa protein, shares a high degree of homology with N-CoR: the C-terminus of SMRT has 48% similarity with the RID of N-CoR

and N-termini of these proteins share 44% similarity. Analogous to N-CoR/RIP13, SMRT/TRAC2 binds to the hinge region of nuclear hormone receptors in the absence of ligand. It also interacts with antihormone-bound steroid hormone receptors, orphan receptors and histone deacetylases (Chen and Evans, 1995; Smith et al., 1997; Nagy et al., 1997 and references therein).

2.2.1.3. TIF-1s. Transcription intermediary factor 1 (TIF1α), a 112-kDa protein, is a RING finger protein that contains a Cys/His cluster (PHD finger), a bromo-related domain, and a coiled-coil domain, a domain responsible for mediating protein–protein interactions. TIF1 interacts with nuclear hormone receptors in a hormone-dependent manner and down-regulates RXRα-, RAR-, and ER-mediated transcriptional activities. Based on the observation that TIF1α interacts with mHP1 and mMOD1, the mouse homologs of the *Drosophila* heterochromatin protein-1, it has been suggested that TIF1 affects the transcriptional activity of nuclear hormone receptors by inducing chromatin remodeling. A second member of the TIF-1 family, TIF1β, also known as KAP-1 (KRAB-associated protein-1), is a 92-kDa protein that is highly homologous to TIF1α. LIke TIF1α, TIF1β recruits mHP1 and mMOD1. In addition, it also interacts with the KRAB domain of human zinc finger factor KOX1/ZNF10. TIF1β represses transcription when fused to a heterologous DNA binding domain, but conversely activates transcription by GR and C/EBPα at the acid glycoprotein promoter. Given these data, it may be that TIF1β represses or activates transcription in a context specific manner through its interaction with chromatin-remodeling proteins, as has been proposed for TIF1α (Le Douarin et al., 1998 and references therein).

2.2.1.4. SUN-CoR. Small unique nuclear receptor corepressor (SUN-CoR), is a highly basic 16-kDa nuclear protein. It enhances transcriptional repression function of TR and the orphan receptor RevErb and contains an intrinsic silencing domain. In addition to interacting with receptor, SUN-CoR recruits SMRT and NCoR, suggesting that these three corepressors may be part of a final corepressor complex devoted to transcriptional repression by nuclear hormone receptors.

2.2.1.5. NSD1. The 285-kDa nuclear receptor-binding SET-domain containing protein 1 (NSD1), differs from other corepressors in several respects. It possesses both repression and activation functions, and contains an SET domain, a motif found in proteins that differentially modulate chromatin structure depending on developmental context. NSDI contains two nuclear receptor interacting domains, NID^{-L} and NID^{+L}. Whereas the NID^{+L} domain of NSDI interacts with receptors when they are bound to hormone, NID^{-L} interacts with the hinge region of RAR and TR in the absence of hormone. The NID^{+L} domain of NSDI interacts with the helix 12 of the receptor, a region known to be required for binding of AF-2 coactivators such as SRC family members.

2.2.1.6. SSN6/TUP1. SSN6/TUP1 is a general corepressor in yeast required for glucose-dependent repression of several genes that also acts as a corepressor for nuclear hormone receptors. The evidence for this comes from mutant yeast strains that lack SSN6/TUP1. The activities of ER and PR are enhanced in these SSN6 null mutant yeast over their activities in wild-type yeast. Similarly, the activity of the antihormone-bound receptor is also potentiated in mutant yeast, suggesting a possible role of SSN6/TUP1 as a corepressor of nuclear hormone receptors.

2.2.1.7. SIN3. SIN3 (RPD-1) is a 105-kDa protein that contains four paired amphipathic helices (PAH) motifs known to be important for protein–protein interaction. Disruption of the *sin3* locus in yeast suggests that it negatively regulates the yeast HO (mating type switching) gene as well as the activity of PR in yeast (Nawaz et al., 1994). Cloning of the mammalian homologs of SIN3, mSIN3a and mSIN3b, has confirmed the role of SIN3 in gene repression. Mammalian SIN3 interacts with a complex containing the Mad and Max transcription factors and represses transcription of their target genes. In addition, a number of recent studies suggest that SIN3 represses the transcriptional activity of nuclear hormone receptor and other transcription factors by recruiting histone deacetylase activity to the promoter of target genes.

2.2.2. KRAB Corepressors

The *K*ruppel *a*ssociated *b*ox (KRAB) domain is found in many human zinc finger proteins. This domain consists of ~75 amino acids and can be divided into two subdomains, an A-box and a B-box. The A-box domain is present in every KRAB domain and is required for transcriptional repression by KRAB domain-containing proteins. The KRAB domain represses transcription of target genes by associating with the corepressor proteins KAP-1 and KRIP-1.

2.2.2.1. KAP-1. *KRAB* *a*ssociated *p*rotein 1 (KAP-1) is an 89-kDa protein that interacts with the

KRAB domain of human zinc finger proteins and represses transcription. The KRAB interaction domain of KAP-1 contains coiled-coil and B-box motifs, suggesting that these motifs may mediate the KRAB–KAP-1 interaction. Like nuclear hormone receptor corepressors, KAP-1 also contains a silencing domain that is separable from the KRAB interacting domain.

2.2.2.2. KRIP-1. *KR*AB-A *i*nteracting *p*rotein 1 (KRIP-1) is an 92-kDa protein that interacts with the KRAB-A region of human zinc finger proteins and modulates the transcriptional repression activity of this region. KRIP-1 is highly homologous to the TIF1s and is a member of the B-boxes coiled-coil subfamily of the RING finger proteins. In addition, KRIP-1 contains an intrinsic silencing domain.

2.2.3. Universal Corepressors

This group contains corepressors that repress general transcription rather than promoter-specific transcription. An example is DR1, a 19-kDa protein that interacts with TBP and recruits a complex containing the DR1-associated protein DRAP-1 to the promoter region of the target gene. DR1 also interacts with the transcription repressor E4BP4 protein and modulates its repressor function. E4BP4 mutants that fail to interact with DR1 are also deficient in their transcription repression function.

2.2.4. Other Corepressors

2.2.4.1. NABs. A 63-kDa protein, NAB1, specifically interacts with and represses transcription of the zinc finger protein NGFIA and the closely related proteins Krox20 and NGFIC. However, NAB1 has no effect on the transcriptional activity of Egr3/NGFIG. The silencing domain of NAB1 is localized to a NAB conserved domain 2 (NCD2), a region found in the C-terminal region of all NAB proteins. NAB2 is another member of the evolutionary conserved family of NAB corepressors that repress transcription of NGFIA and Krox20. NAB2 is a 58-kDa protein that, as with NAB1, contains a silencing domain localized within the NCD2 domain of the protein.

2.2.4.2. Groucho. Groucho (Gro) is a 79-kDa protein that interacts with and mediates repression by the Hairy bHLH proteins, the Engrailed homeodomain proteins, and high mobility group proteins such as TCF/LEF-1. Gro contains multiple tandem repeats of the WD-40 repeat motif, known to mediate protein–protein interaction. In *Drosophila*, Gro converts Dorsal from an activator to a repressor and plays a critical role in the dorsal/ventral patterning system. Gro also represses Wingless/Wnt signaling activity by interacting with both TCF/LEF-1 in both *Xenopus* and *Drosophila* systems.

2.2.4.3. HIPKs. *H*omeodomain *i*nteracting *p*rotein *k*inases (HIPKs) are proteins that interact with NK-homeodomain transcription factors and modulate their repressor function. HIPKs contain a conserved protein kinase domain, a homeodomain interaction domain, and an N-terminal silencing domain. At present, the role of protein kinase activity of the HIPKs in gene repression is not clear.

2.3. Molecular Mechanism of Action of Corepressors

2.3.1. Interference and/or Sequestration

This mechanism involves specific protein–protein interactions between corepressor proteins and the proteins of the transcription initiation complex. The interaction of the corepressor with the components of the transcription initiation complex may inhibit or destabilize preinitiation complex assembly by either sequestering target protein(s) from the PIC or by physically blocking the assembly of the complex. Recruitment of TFIIB is a critical step in the assembly of the PIC and is required for the recruitment of RNA polymerase II into this complex. N-CoR has been shown to interact with the basal transcription factor TFIIB and may act to sequester TFIIB from the preinitiation complex, thereby potentially blocking a critical step in its assembly.

2.3.2. Covalent Modification

This mechanism involves recruitment of histone deacetylases (chromatin remodeling enzymes) by corepressors to the promoter region of the target genes, thus compacting nucleosomes into transcriptionally inert chromatin by promoting the deacetylation of histones. The supporting evidence for the involvement of histone deacetylation in gene repression is provided by numerous recent studies. These studies suggest that nuclear corepressors, SMRT/N-CoR, SIN3, and the histone deacetylases form a multiprotein complex that is essential for gene repression. Furthermore, inhibitors of histone deacetylases, such as trichostatin A (TSA), and antibodies against N-CoR, SIN3, or histone deacetylases relieve the repression, further confirming the role of these proteins in recruiting histone deacetylases to effect gene repression (Heinzel et al. 1997; Nagy et al., 1997). Interestingly, CBP, a HAT previously characterized as a gen-

eral coactivator, targets TCF for acetylation, thereby uncoupling its interaction with its coactivator, Armadillo, and repressing the Wnt/Wingless signaling pathway. The clear inference in this case is that acetylation and deacetylation are not intrinsically positive or negative stimuli for transcription, but rather that their effects are context specific.

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354 Characterization of a liganded progesterone receptor-associated complex

Neil J. McKenna, Ming-Jer Tsai, Sophia Y.Tsai and Bert W. O'Malley. Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030.

Transcriptional regulation by activated nuclear receptors occurs as a result of their interaction with multiprotein complexes containing nuclear receptor coactivators. Biochemical purification using an affinity-tagged progesterone receptor (PR) indicated its interaction with a complex of high molecular size polypeptides, including the SRC family members SRC-1 and RAC-3/SRC-3. The nuclear receptor cointegrator, CREB-binding protein (CBP), was not present in the liganded PR complex. Our results support the hypothesis that activated nuclear receptors interact with coactivator complexes on a modular basis, with differing degrees of affinity for different complexes. Further studies will identify unknown members of this complex, and should yield important information on the functional properties of the PR complex. This work was supported in part by a United States Army Breast Cancer Postdoctoral Fellowship (NJMcK).

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f. Harris, Michael
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GEOM), Institute for
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cat@cmcb.uq.edu.au

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356 A Novel Role of the Basal Transcription Factor NF-Y to Co-recruit Transcription Coactivator ASC-2 with Thyroid Hormone Receptor.

Soon-Young Na, Soo-Kyung Lee and Jae Woon Lee, Center for Ligand and Transcription, Chonnam National University, Kwangju 500-757, Korea

We have recently isolated a transcription coactivator of nuclear receptors (designated ASC-2) that is amplified in certain human cancers. In an effort to understand its role in transcriptional coactivation, we isolated a series of ASC-2-interacting factors by using the yeast two hybrid assay. Surprisingly, these included NF-Yc, a component of the

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